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Decreased Arteriolar Tetrahydrobiopterin is Linked to Superoxide Generation from Nitric Oxide Synthase in Mice Fed High Salt

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Abstract

Objective—Impaired endothelium-dependent arteriolar dilation in mice fed high salt is due to local oxidation of nitric oxide (NO) by superoxide anion (O_2^-). We explored the possibility that “uncoupled” endothelial nitric oxide synthase (eNOS) is the source of this O_2^- .

Methods—Levels of L-arginine (L-Arg), tetrahydrobiopterin (BH_4) and O_2^- (hydroethidine oxidation) were measured in spinotrapezius muscle arterioles of mice fed normal salt (0.45%, NS) or high salt (4%, HS) diets for 4 weeks, with or without dietary L-Arg supplementation. The contribution of NO to endothelium-dependent dilation was determined from the effect of N^ω -nitro-L-arginine methyl ester (L-NAME) on responses to acetylcholine (ACh).

Results—Arterioles in HS mice had lower $[BH_4]$ and higher O_2^- levels than those in NS mice. ACh further increased arteriolar O_2^- in HS mice only. L-Arg supplementation prevented the reduction in $[BH_4]$ in arterioles of HS mice, and O_2^- was not elevated in these vessels. Compared to NS mice, arteriolar ACh responses were diminished and insensitive to L-NAME in HS mice, but not in HS mice supplemented with L-Arg.

Conclusions—These findings suggest that eNOS uncoupling due to low $[BH_4]$ is responsible for O_2^- generation and reduced NO-dependent dilation in arterioles of mice fed a high salt diet.

Keywords

microcirculation; dietary salt; nitric oxide; tetrahydrobiopterin; superoxide anion; L-arginine; mouse

Introduction

High dietary salt intake, even in the absence of hypertension, can result in impaired arteriolar responses to hypoxia [20], transmural (myogenic) stretch [31], and vasoactive stimuli that normally rely on endothelial nitric oxide (NO) release [4,5,17-19]. Because metabolic, myogenic, and endothelium-dependent stimuli can all play a role in the moment-to-moment regulation of tissue blood flow, these salt-dependent changes in arteriolar function can have profound physiological relevance [6,21].

Our previous studies indicate that impaired NO-dependent responses in rats and mice fed high salt are due to oxidation of NO in the arteriolar wall by superoxide anion (O_2^-) or one of its immediate metabolites [17,18,32]. A strong candidate for the source of this O_2^- is endothelial nitric oxide synthase (eNOS), which can generate large amounts of O_2^- instead of NO if the normal flow of electrons from its heme core to L-Arginine (L-Arg) is diverted to molecular oxygen --a condition widely referred to as “uncoupling” [8,37]. In support of this possibility, we have recently found that in mice fed high salt, NOS inhibition completely abolishes steady-state arteriolar O_2^- accumulation and also prevents the increase in these O_2^- levels that can be triggered by ACh [32].

Tetrahydrobiopterin (BH_4) is an essential cofactor for eNOS, and as such BH_4 bioavailability plays a central role in the regulation of endothelial NO production. An increase in BH_4 synthesis or availability leads to increased NO production [2,9,25], whereas a reduction in BH_4 availability is one of the primary causes of eNOS uncoupling [8,37]. In the current study, we studied the relationships among dietary salt, vascular wall BH_4 and O_2^- levels, and NO-dependent dilation to further investigate the contribution of uncoupled eNOS to the impaired endothelium-dependent dilation of arterioles after ingestion of a high salt diet.

Materials and Methods

All surgical and experimental procedures were approved by the West Virginia University Animal Care and Use Committee. Ten-week old inbred male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were placed on a whole-grain diet containing either 0.45% NaCl (normal salt, NS) or 4% NaCl (high salt, HS) (TD88311 and TD92100 diets; Teklad, Madison, WI). We have previously shown that these mice remain normotensive when placed on a high salt diet [30]. In subsets of mice from each dietary group, the drinking water also contained L-Arg (1.51% arginine-HCl). Dietary L-Arg supplementation by this method has been found to increase not only endothelial cell L-Arg levels, but also endothelial BH_4 levels, leading to increased NO synthesis [13]. Other mice received drinking water containing L-alanine (2.55%) to serve as isonitrogenous controls for the mice receiving L-Arg. All mice were studied 4-5 weeks after being placed on their respective diets/drinking water regimens.

Assessment of arteriolar wall superoxide levels and endothelium-dependent dilation

Each mouse was anesthetized with sodium thiopental (50 mg/kg, i.p.) and placed on a heating pad to maintain a 37°C core temperature. The right spinotrapezius muscle was exteriorized for microscopic observation leaving all innervation and feed vessels intact and undisturbed. Throughout this surgery and subsequent procedures, the muscle was continuously superfused with an electrolyte solution (119 mM NaCl, 25 mM $NaHCO_3$, 6 mM KCl and 3.6 mM $CaCl_2$) warmed to 35°C and equilibrated with 95% N_2 -5% CO_2 (pH=7.35-7.40). Superfusate flow rate was maintained at 4-6 ml/min to minimize equilibration with atmospheric O_2 [7]. After muscle exteriorization, the mouse was transferred to the stage of an Olympus BHMJ microscope (Hyde Park, NY) fitted with a color CCD video camera (Dage-MTI, Michigan City, IN). Images were displayed on a high-resolution video monitor and stored on videotape for later analysis. Observations were made at final magnification of 1460X. For assessment of endothelium-dependent dilation, arteriolar inner diameters were measured during videotape replay with a video caliper (Cardiovascular Research Institute, Texas A&M University).

Arteriolar wall O_2^- levels were assessed by the hydroethidine (HE) oxidation assay [21,32]. Because it is not oxidized by H_2O_2 , HOCl, ONOO⁻ or O_2 , HE is considered to be relatively specific for the detection of O_2^- [3]. HE permeates cell membranes and, when oxidized by

O_2^- , is converted to fluorescent ethidium bromide. HE (1×10^{-3} M) was added to the superfusate, and the muscle was continuously exposed to this solution for 30 minutes in complete darkness to prevent HE photo-destruction. In some preparations, the effect of increased eNOS activity on O_2^- production was assessed by also adding 1×10^{-5} M ACh to the HE-containing superfusate. After this HE loading period, the muscle was rinsed with the normal superfusate for 15 minutes to remove extracellular HE before measuring fluorescence levels. Each vessel was selected for measurement using transillumination and standard brightfield microscopy, with the focal plane set at the center of the vessel. The inner (luminal) and outer edges of the vessel wall were clearly visible at the magnification we used (see above), but the practical resolution of our system under in-vivo conditions did not permit a clear distinction between the endothelium and adjacent smooth muscle layers. An image of the vessel was obtained during brief (1 s) epi-illumination of the muscle with a mercury lamp, using excitation and emission filters for detection of ethidium bromide fluorescence (480-550 nm bandpass, 590 nm barrier). Using a photometric window measuring $1 \times 10 \mu\text{m}$ relative to the microscopic field, measurements of average pixel intensity (0-255 grey scale) were made at a series of 10 sites along each vessel wall. These measurements were then used to calculate a mean value for each vessel. Tissue autofluorescence at this wavelength was measured before HE exposure, and then subtracted from signals measured after HE exposure to determine specific ethidium bromide fluorescence intensities. All images were acquired, stored and analyzed with Metamorph 6.01 imaging software (Universal Imaging Corporation, Downingtown, PA).

To determine the effect of high salt intake on endothelium-dependent dilation, we evaluated arteriolar responsiveness to iontophoretically-applied acetylcholine (ACh; Sigma Chemical, St. Louis, MO) in each dietary group. Since L-alanine was found to have no effect on ACh-stimulated O_2^- generation (see Results), these experiments were conducted only on mice consuming normal or L-Arg-supplemented drinking water. A beveled micropipette (2-3 μm tip diameter) was filled with 0.025 M ACh in distilled water and positioned with its tip in light contact with the arteriolar wall. A retaining current of 70-100 nA was used to prevent passive diffusion of ACh from the micropipette tip during the control period, and net ejection currents of 5, 20, and 80nA (delivered in random order) were used for ACh application. For each level of ACh, the vessel was continuously observed during a 1-min control period, a 2-min application period, and a minimum 2-min recovery period. To determine the contribution of NO to these endothelium-dependent responses, the NOS inhibitor N^ω -nitro-L-arginine methyl ester (L-NAME) was then infused into the superfusate delivery line to produce an L-NAME concentration of 10^{-4} M in the muscle bathing solution. After a 10-min equilibration period, arteriolar responses to all levels of ACh were reassessed, with L-NAME superfusion continued throughout to ensure maximal NOS inhibition. Although we used N^G monomethyl L-arginine (L-NMMA) to inhibit eNOS in our earlier study [32], we used L-NAME in the current study because it is more effective inhibitor of eNOS-mediated O_2^- production due to its ability to completely antagonize the transfer of electrons to oxygen as well as L-arginine [29].

In other mice from each group, the intrinsic responsiveness of arteriolar smooth muscle to NO was evaluated by iontophoretically applying the NO donor sodium nitroprusside (SNP, Sigma) to the arteriolar wall. Beveled micropipettes were filled with 0.05 M SNP in distilled water, retaining currents of up to 100 nA were used to prevent passive diffusion, and net ejection currents of 5, 10 and 20nA were used to apply SNP (1-min control, 2-min application, minimum 2-min recovery).

Assessment of vascular wall L-Arginine and biopterin levels

Arcade arterioles were quickly excised from the spinotrapezius muscle with the aid of a dissecting microscope, placed in a cryovial containing 50 μl of 5 mM dithioerythritol

(deoxygenated using helium), and then snap frozen in liquid nitrogen. For each sample, vessels from the muscles of 6 mice were pooled to obtain enough tissue for analysis. To determine the effect of increased dietary salt intake and/or chronic L-Arg supplementation on local BH₄ and arginine availability, arteriolar BH₄ and arginine levels were measured by reverse-phase HPLC with fluorescence detection, as previously described [24]. To determine if any differences in arteriolar [BH₄] among groups could be due to a change in BH₄ metabolism, arteriolar [BH₂] was also measured as biopterin level in base oxidation [24] for calculation of the [BH₂]/[BH₄] ratio in arterioles of each group. This ratio is widely used as an index of BH₄ oxidation; the immediate product of BH₄ oxidation is quinonoid-7,8-dihydrobiopterin (qBH₂), but this species is extremely unstable and spontaneously rearranges to yield 7,8-dihydrobiopterin (BH₂) and 7,8-dihydropterin [34]. Briefly, 10 µg of arteriolar tissue were homogenized in 0.1 ml of 0.1 M phosphoric acid containing 5 mM dithioerythritol and 15 µl of 2 M trichloacetic acid (TCA). BH₄ standard (50 pmol/ml) or cell extract (50 µl) was mixed with 15 µl of 0.2 M TCA and 15 µl of acidic oxidizer (1% I₂/2% KI in 0.2 M TCA) (acidic oxidation) or with 15 µl of 1 M NaOH and 15 µl of alkaline oxidizer (1% I₂/2% KI in 3 M NaOH) (alkaline oxidation). After 1-h incubation at 25° C in the dark, excess iodine was removed by adding 25 µl of 20 mg/ml ascorbic acid. After neutralization, 50 µl of the solution was analyzed on a Phenosphere 5 ODS-1 column (4.6 mm × 25 cm, 5 µm) using isocratic elution (flow rate of 1 ml/min) and fluorescence detection (excitation 350 nm and emission 440 nm). The mobile phase solvent was 5% HPLC-grade methanol, 95% HPLC-grade water, 7.5 mM sodium phosphate, pH 6.35 (15 min running time).

The amount of BH₂ in tissue extracts was determined from the amount of biopterin measured after alkaline oxidation, and the amount of BH₄ in tissue extracts was determined by subtracting the amount of biopterin measured after alkaline oxidation from the amount of biopterin measured after acidic oxidation [24].

Measurement of vascular wall GTP cyclohydrolase-1 expression

To determine if any differences in arteriolar wall [BH₄] among groups could be due to differences in BH₄ synthesis, we used Western analysis to determine the content of GTP cyclohydrolase-1 (GTPCH), the rate-controlling enzyme for BH₄ biosynthesis [34]. Arterioles were quickly excised from the spinotrapezius muscle and snap frozen in liquid nitrogen. For each sample, vessels from the muscles of 5-6 mice were pooled to obtain enough tissue for Western blotting. Arteriolar protein was extracted and loaded on 9.5–16% polyacrylamide gels. Separated proteins were blotted onto nitrocellulose and the blots were blocked with 5% non-fat dried milk in Tris-buffered saline with 0.1% Tween 20 (blocking buffer). For analysis of GTPCH, primary antibody (prepared against purified rat recombinant GTPCH) was diluted 1:10000 in blocking buffer, and the secondary antibody, peroxidase-conjugated donkey anti-rabbit IgG, was used at 1:100000 in blocking buffer. Bands were visualized with a chemiluminescence signal enhancer and exposed to autoradiography film. Blots were digitized and optical density measurements made with BioRad Multi-Analyst software.

Results

After HE exposure, control ethidium bromide fluorescence was significantly greater in arterioles of untreated HS mice (mean pixel intensity: 210±5) than in those of untreated NS mice (mean pixel intensity: 155±2) (Figure 1). In NS mouse arterioles, L-Arg supplementation had no effect on control fluorescence, and ACh had no effect on fluorescence regardless of whether the mice were untreated or supplemented with L-Arg. In contrast, ACh significantly increased arteriolar fluorescence in untreated HS mice (to 234±5). In HS mice supplemented with L-Arg, control arteriolar fluorescence (153±3) was

significantly lower than that in untreated HS mice, and not significantly different from the control values measured in NS mice (either untreated or L-Arg supplemented). Furthermore, ACh did not increase arteriolar fluorescence in HS mice supplemented with L-Arg (159 ± 3).

Supplementation with L-alanine had no effect on arteriolar ethidium bromide fluorescence in NS mice, and in contrast to L-Arg, did not prevent the ACh-induced fluorescence increase in HS mice (data not shown). Because L-alanine did not mimic the effects of L-Arg in arterioles of HS mice, we concluded that the effect of L-Arg in these animals was specific and not due to elevated nitrogen intake. L-alanine supplementation was therefore discontinued for all subsequent experiments.

As shown in Figure 2, there was no difference between untreated NS and HS mice in arteriolar wall L-Arg content (97.5 ± 3.0 and 93.3 ± 3.8 fmol/mg, respectively), and L-Arg supplementation increased arteriolar L-Arg content by the same amount in both groups (NS: to 155.5 ± 4.7 fmol/mg, a 59% increase; HS: to 145.4 ± 3.1 fmol/mg, a 56% increase).

As shown in Figure 3, arteriolar wall BH₄ content was significantly lower in untreated HS mice than in untreated NS mice (38.7 ± 1.6 vs. 70.0 ± 3.1 fmol/mg). However, whereas L-Arg supplementation had no effect on arteriolar BH₄ in NS mice, it increased BH₄ content in HS mice to a level that was not significantly different from that in untreated NS mice (65.0 ± 2.7 fmol/mg). The ratio of [BH₂] to [BH₄] was much higher in arterioles from untreated HS mice than in those from untreated NS mice (0.275 ± 0.015 vs. 0.074 ± 0.005) (Figure 4). However, whereas L-Arg supplementation had no effect on this ratio in NS mice, it returned the ratio in HS mice almost to the same level as that in NS mice (0.102 ± 0.006). In contrast to biopterin levels, there was no difference between untreated NS and HS mice in arteriolar wall GTPCH expression, and L-Arg supplementation had no effect on this expression in either dietary group (Figure 5).

Under control conditions *in vivo*, there were no significant differences among diet/treatment groups in resting arteriolar diameters (NS untreated: 31 ± 3 μ m, NS+L-Arg: 34 ± 2 μ m, HS untreated: 33 ± 2 μ m, HS+L-Arg: 29 ± 1 μ m). Iontophoretically-applied ACh caused arteriolar dilation in all groups, but for each current-dose of ACh, the magnitude of this dilation was significantly lower in untreated HS mice than in untreated NS mice ($5\pm 3\%$ vs. $15\pm 4\%$ for 5 nA ACh, $9\pm 2\%$ vs. $42\pm 6\%$ for 20 nA ACh, and $16\pm 4\%$ vs. $52\pm 8\%$ for 80nA ACh) (Figure 6). However, although L-Arg supplementation had no significant effect on ACh responses in NS mice, it restored ACh-induced arteriolar dilation to normal in HS mice ($12\pm 4\%$, $36\pm 6\%$ and $48\pm 8\%$ dilation for 5, 20 and 80 nA ACh, respectively).

Exposure to L-NAME had no significant effect on resting arteriolar diameters in any experimental group (NS untreated: 29 ± 2 μ m, NS+L-Arg: 34 ± 2 μ m, HS untreated: 29 ± 2 μ m, HS+L-Arg: 29 ± 1 μ m). However, as shown in Figure 7, L-NAME greatly reduced arteriolar dilation to all levels of ACh in NS mice, decreasing the magnitude of the dilation by 72-90%. L-Arg supplementation in these animals did not appreciably change the effect of L-NAME on responses to ACh, with the magnitude of dilation being decreased by 64-84%. In contrast to NS mice, arteriolar dilation to ACh was not sensitive to L-NAME in untreated HS mice. However, in HS mice supplemented with L-Arg, L-NAME reduced the magnitude of arteriolar responses by an amount similar to that found in NS mice (58-84%).

As shown in Figure 8, iontophoretic application of SNP caused a dose-dependent dilation of arterioles in all groups, but there were no significant differences among any of the groups in the magnitude of this dilation for any level of SNP application.

Discussion

The major findings of this study are as follows: (1) Arterioles in HS mice have lower BH₄ levels and higher O₂⁻ levels than those in NS mice. (2) ACh, which increases eNOS activity, further increases arteriolar O₂⁻ in HS mice only. (3) L-Arg supplementation prevents the reduction in BH₄ in arterioles of HS mice, and these vessels do not have elevated O₂⁻ levels. (4) ACh-induced arteriolar dilation is smaller in HS mice than in NS mice, but not if HS mice are supplemented with L-Arg. Collectively, these findings suggest that eNOS uncoupling due to low BH₄ bioavailability is responsible for O₂⁻ generation and reduced endothelium-dependent dilation in arterioles of mice fed a high salt diet.

Previous studies have documented that reduced BH₄ bioavailability, leading to uncoupling of eNOS and O₂⁻ production, contributes importantly to vascular dysfunction in various other pathological states, including hypertension, diabetes, and chronic alcohol consumption [1,9,10,13,15,25,27,36]. In an earlier study on the mouse spinotrapezius muscle, we found that ingestion of a high salt diet leads to arteriolar O₂⁻ accumulation and that the NOS inhibitor L-NMMA prevents this O₂⁻ accumulation, implicating uncoupled eNOS as the source of this O₂⁻ [32]. This interpretation is further supported by our current finding that arteriolar wall BH₄ levels in HS mice are almost 50% lower than those in NS mice (Figure 3), and that preserving normal BH₄ levels in HS mice by dietary L-Arg supplementation is as effective as NOS inhibition in preventing arteriolar O₂⁻ accumulation (Figure 1).

In some cases, reduced BH₄ bioavailability associated with vascular dysfunction is the result of insufficient de novo production due to reduced expression of GTPCH [25,27]. In other cases, it is due to accelerated BH₄ oxidation by reactive oxygen species [15,16]. In the current study, we found no difference between NS and HS mice in GTPCH expression (Figure 5), suggesting that BH₄ production may not have been reduced in HS mice. However, we did find that BH₂, the stable oxidation product of BH₄, is profoundly elevated in arterioles of HS mice (Figure 4), which indicates that the reduced BH₄ levels are due at least in part to increased BH₄ oxidation. The current study provides no direct evidence for the identity of the reactive molecule(s) responsible for accelerated BH₄ oxidation in arterioles of HS mice. We and others have consistently documented the presence of elevated arteriolar O₂⁻ in both mice and rats fed high salt diets [17-19,32,43]. However, O₂⁻ is a relatively poor oxidizer of BH₄ [16]. A more likely candidate is peroxynitrite (ONOO⁻), which is formed when O₂⁻ interacts with NO and is a strong oxidizer of BH₄ [14,16].

Administration of exogenous L-Arg has previously been found to lessen or reverse endothelial dysfunction in some cardiovascular disease states [11,33,42], and our current findings are consistent with recent evidence that at least part of this effect is due to an increase in BH₄ availability. In both normal and diabetic rats, dietary L-Arg supplementation leads to an increased plasma concentration of L-Arg, increased endothelial cell concentrations of L-Arg and BH₄, and increased endothelial NO synthesis [13]. Studies on cultured endothelial cells indicate that increased cellular L-Arg increases intracellular BH₄ levels by increasing the expression of GTPCH [41]. We were able to increase arteriolar wall L-Arg levels by the same amount in both dietary groups by providing supplemental L-Arg in the drinking water (Figure 2). However, this led to increased BH₄ levels only in the arterioles of HS mice (Figure 3), and this occurred through a reduction in BH₄ oxidation rather than an increase in GTPCH expression (Figures 4 and 5). The reason for the difference between our study and the previous study on endothelial cells is not clear, but it could be attributable to different methodologies (addition of L-Arg to endothelial cells in vitro vs. in vivo supplementation), differences in the duration of exposure to increased L-Arg levels (24-48 hours vs. 4 weeks in our study), or even species differences (bovine vs. murine). L-Arg is an effective scavenger of O₂⁻ [39], and this antioxidant property could be

responsible for the reduced BH₄ oxidation we found in HS mice. Increasing arteriolar wall L-Arg would lead to reduced O₂⁻, and therefore less ONOO⁻ formation. In this scenario, the lack of effect of increased L-Arg on arteriolar BH₄ in NS mice would not be surprising because there is little if any O₂⁻ for the L-Arg to scavenge, and little ONOO⁻ to begin with.

In comparing the values for untreated NS vs. untreated HS mice, it is clear that high salt intake has no effect on endogenous arteriolar wall L-Arg levels (Figure 2). This is consistent with previous findings in Sprague-Dawley rats that ingestion of a high (8%) salt diet has no effect on plasma L-Arg levels [40], and that dietary salt over a wide range has no effect on systemic L-Arg synthesis or on the expression or activity of hepatic L-arginase, an enzyme that plays a central role in the regulation of plasma L-Arg levels [12].

The impact of high dietary salt intake on microvascular function has historically been studied almost exclusively in the rat [6]. We and others have found no contribution of NO to the endothelium-dependent dilation of mesenteric or skeletal muscle resistance vessels in rats fed high salt [4,5,35,43], presumably due to scavenging of NO by the O₂⁻ that accumulates in these vessels [17-19,43]. However, the rat is apparently different from the mouse in that this elevated O₂⁻ is due primarily to increased activity of NADP(H) oxidase and xanthine oxidase [18,43]. Many investigators now use the mouse to explore mechanisms of cardiovascular disease because of the availability of numerous murine models with targeted deletion or overexpression of genes relevant to cardiovascular control. Similar to our previous findings in the rat, our first study in mice fed high salt showed a correlation between increased vascular wall O₂⁻, a loss of NO bioavailability, and blunted endothelium-dependent dilation in skeletal muscle arterioles [32]. The results of the present study confirm that increased arteriolar O₂⁻ is accompanied by a loss of NO in salt-fed mice; arteriolar responses to ACh, which were reduced 70-90 % by L-NAME in NS mice, were much smaller and insensitive to L-NAME in HS mice (Figure 7). The undiminished responses to ACh in HS mice supplemented with L-Arg are apparently due to preservation of the L-NAME-sensitive component of the dilation (Figures 6 and 7), in association with the maintenance of normal vascular wall BH₄ levels and an absence of O₂⁻ accumulation. Dilation of arterioles to the NO donor SNP was identical in all experimental groups (Figure 8), indicating that guanylyl cyclase activity and cGMP-dependent pathways in smooth muscle are not influenced by dietary salt content or by levels of O₂⁻, L-Arg or BH₄ in the arteriolar wall. We therefore conclude that the effectiveness of L-NAME in reducing endothelium-dependent dilation of arterioles in HS mice given L-Arg is due to a preservation of NO bioavailability rather than an increase in arteriolar smooth muscle responsiveness to NO.

During application of ACh at the highest concentration, the residual dilation that persists after L-NAME treatment appears to be greater in NS mice given L-Arg than in untreated NS mice (Figure 7). Because L-Arg and L-NAME compete for the same binding site on eNOS, it is possible that eNOS was not fully inhibited under conditions in which arteriolar wall L-Arg levels were increased almost 60% by supplementation (Figure 2). However, L-NAME appears to have the same effect on dilations to ACh at lower concentrations in both groups, suggesting this may not be a consistent problem.

Because both the L-NAME used in this study and the L-NMMA used in our previous study [32] are nonspecific, they will inhibit the neuronal isoform of NOS (nNOS) as well as eNOS. Although nNOS expression and activity is normally low in mouse arterioles, it can be increased in certain pathological states [23,26]. Because low BH₄ levels can also lead to uncoupling of nNOS [38], we can't rule out the possibility that if nNOS is upregulated in salt-fed mice, this isoform could be the source of at least some of the O₂⁻ generated in arterioles of HS mice.

The impact of the salt-dependent reduction in NO bioavailability on the regulation of muscle blood flow in the mouse is unknown. Nitric oxide contributes importantly to the arteriolar dilation and hyperemia that accompanies muscle contraction in the rat, hamster and mouse [21,22,28,30], and we have previously reported that arteriolar dilation during high frequency contraction of the spinotrapezius muscle is reduced in rats on the same high salt diet regimen as the mice in this study [21]. It is reasonable to expect that active hyperemia (and other forms of local blood flow control that involve NO) would be reduced in mice fed a high salt diet, but additional studies will be required to assess the precise physiological influence of dietary salt on blood flow regulation in the mouse.

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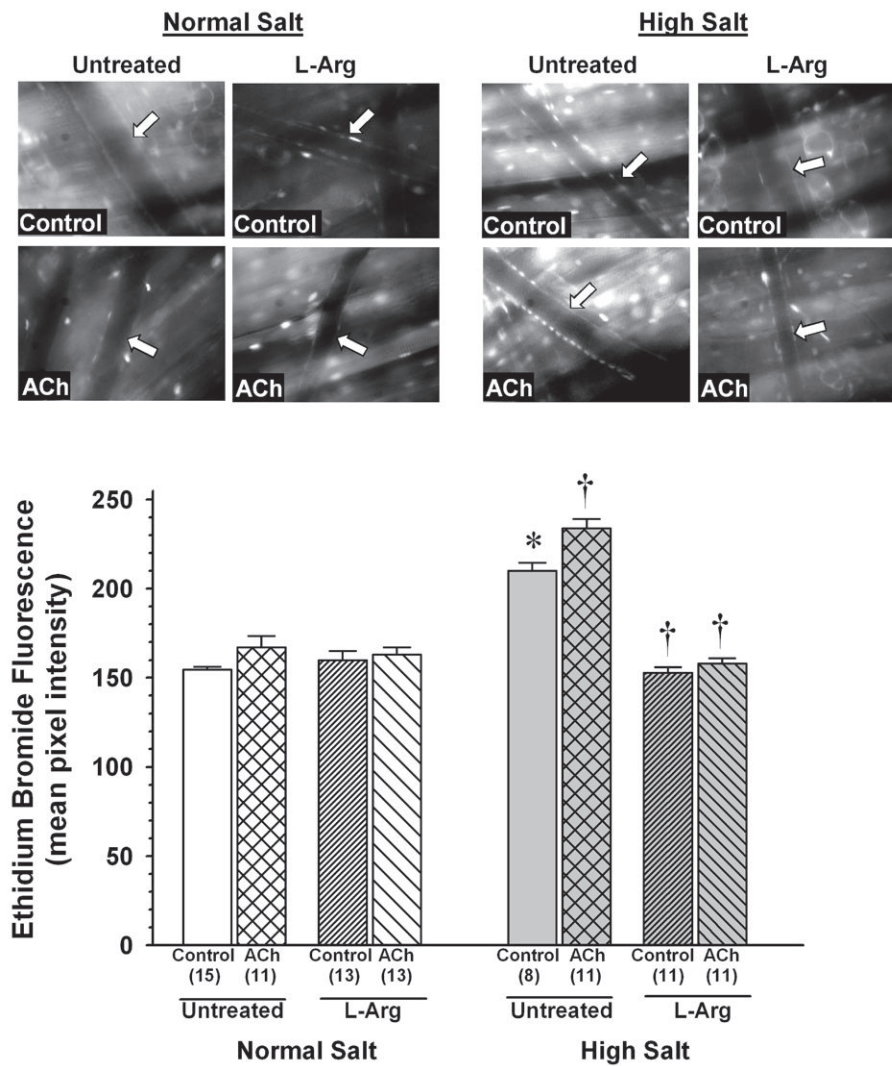


Figure 1. Arteriolar ethidium bromide fluorescence after exposure of the spinotrapezius muscle to hydroethidine in mice fed normal salt or high salt diet, with or without L-arginine (L-Arg) supplementation. Measurements were made in otherwise untreated muscles (Control) or in muscles also exposed to ACh (1×10^{-5} M). Top: representative fluorescence images for each experimental group. Arrow in each image indicates position of vessel wall. Bottom: mean fluorescence values for each group. Values in parentheses represent number of vessels studied in each group. * $P < 0.05$ vs. Control value in untreated NS mice. † $P < 0.05$ vs. Control in same diet group.

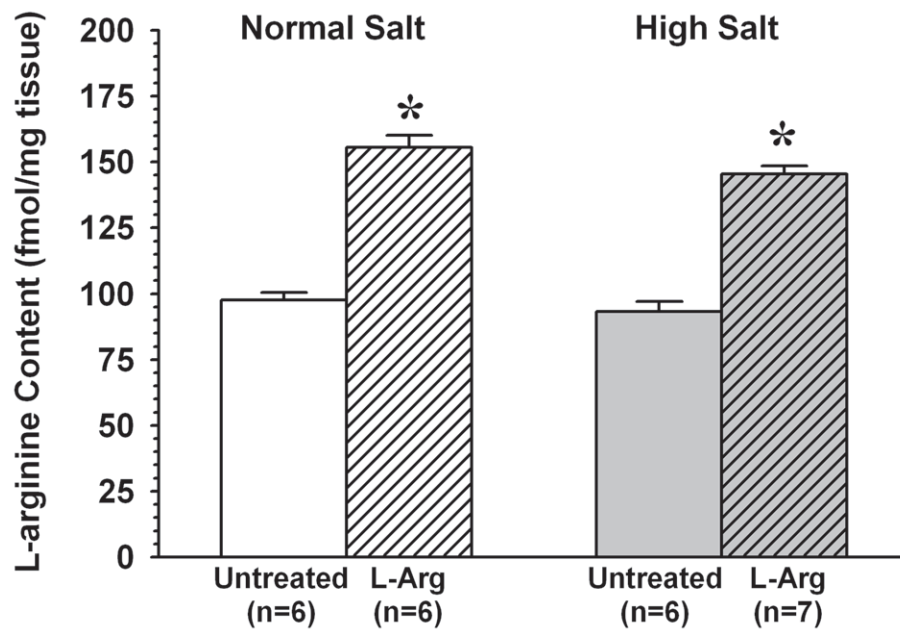


Figure 2. Effect of L-arginine supplementation (L-Arg) on arteriolar wall L-arginine levels in mice fed normal salt or high salt diet. * $P < 0.05$ vs. untreated group. In Figures 2-5, n= number of samples, with each sample comprised of muscle vessels pooled from 5-6 mice.

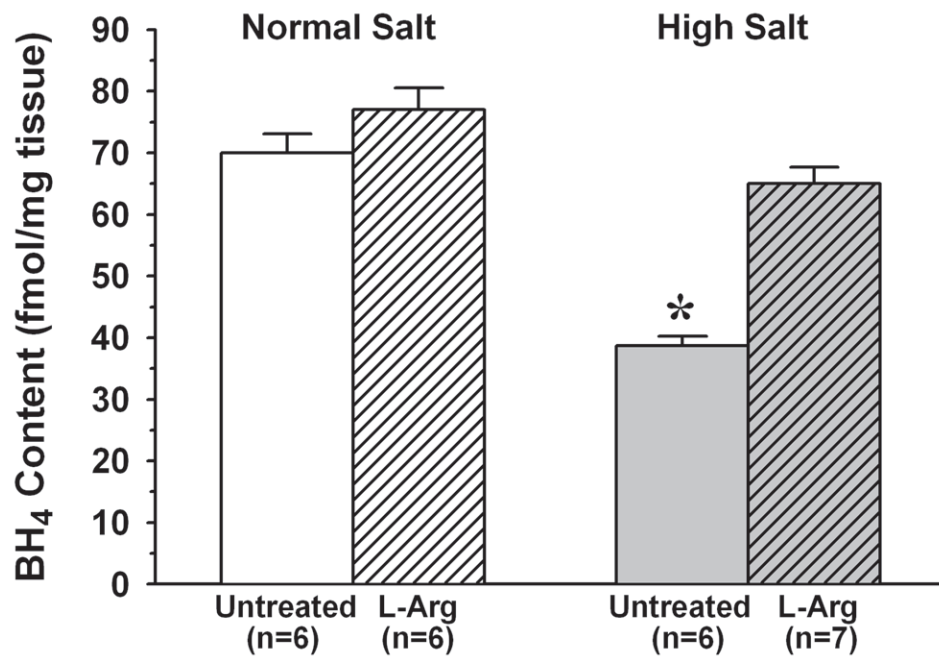


Figure 3. Arteriolar wall BH₄ content in mice fed normal salt or high salt diet, with or without L-Arg supplementation. * P < 0.05 vs. untreated and L-Arg supplemented normal salt mice.

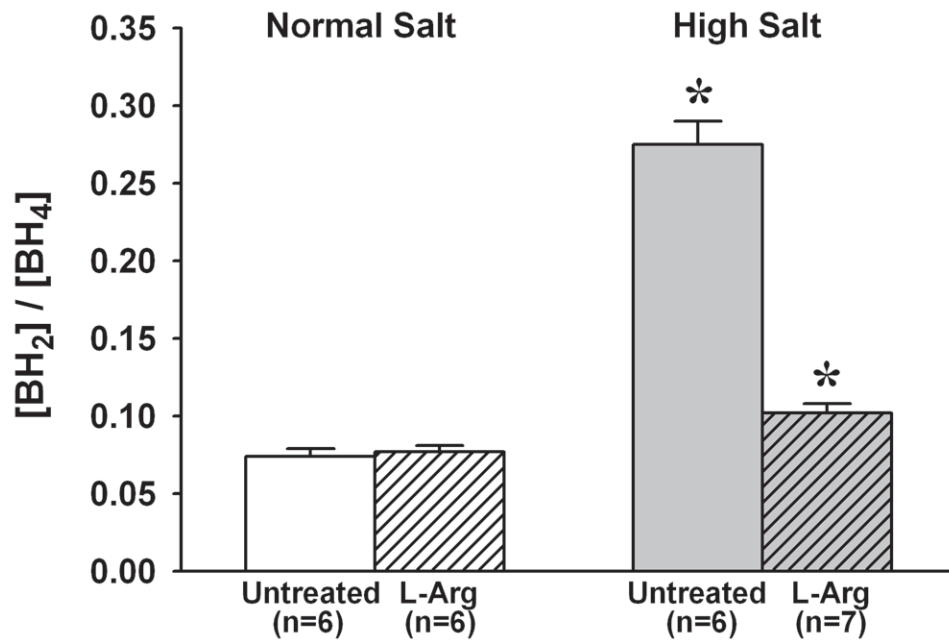


Figure 4. Ratio of BH₂ content to BH₄ content in arterioles from mice fed normal salt or high salt diet, with or without L-Arg supplementation. * P < 0.05 vs. untreated and L-Arg supplemented normal salt mice.

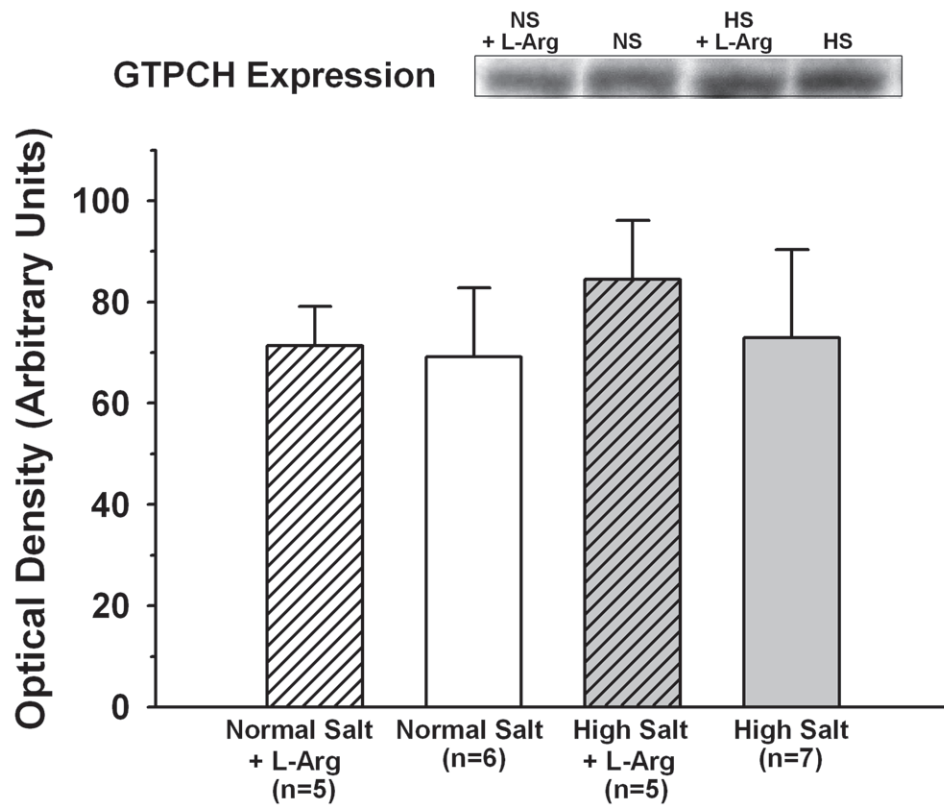


Figure 5. GTP cyclohydrolase-1 expression in arterioles from mice fed normal salt or high salt diet, with or without L-Arg supplementation.

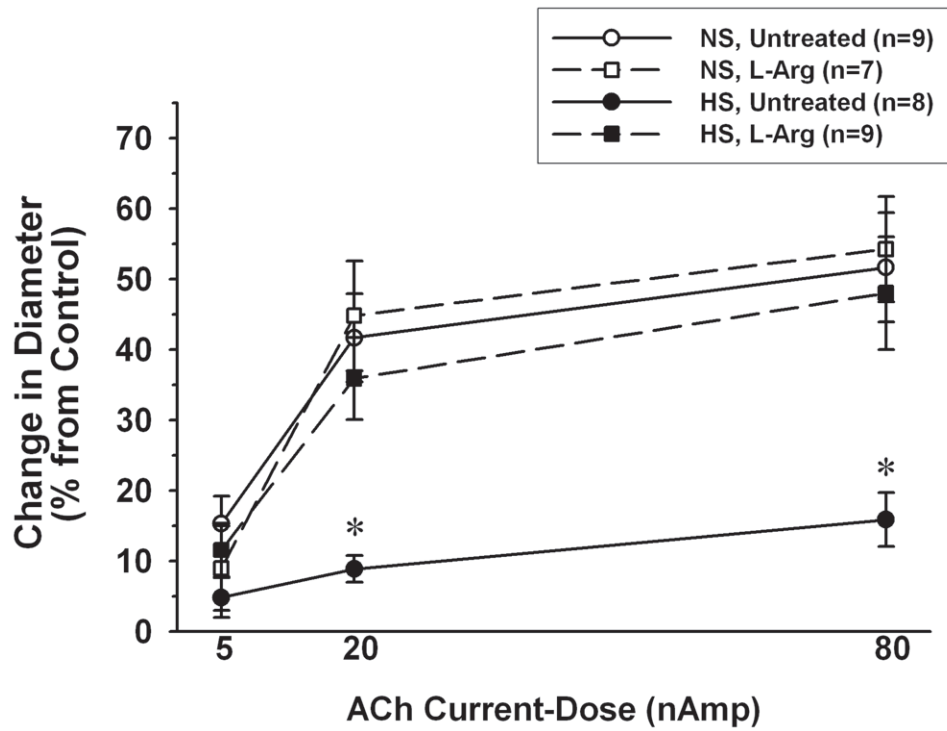


Figure 6. Arteriolar dilation in response to iontophoretic application of ACh in mice fed normal salt (NS) or high salt (HS) diet, with or without L-Arg supplementation. * $P < 0.05$ vs. both NS groups and the HS group supplemented with L-arginine. n= number of vessels (1 vessel studied per mouse).

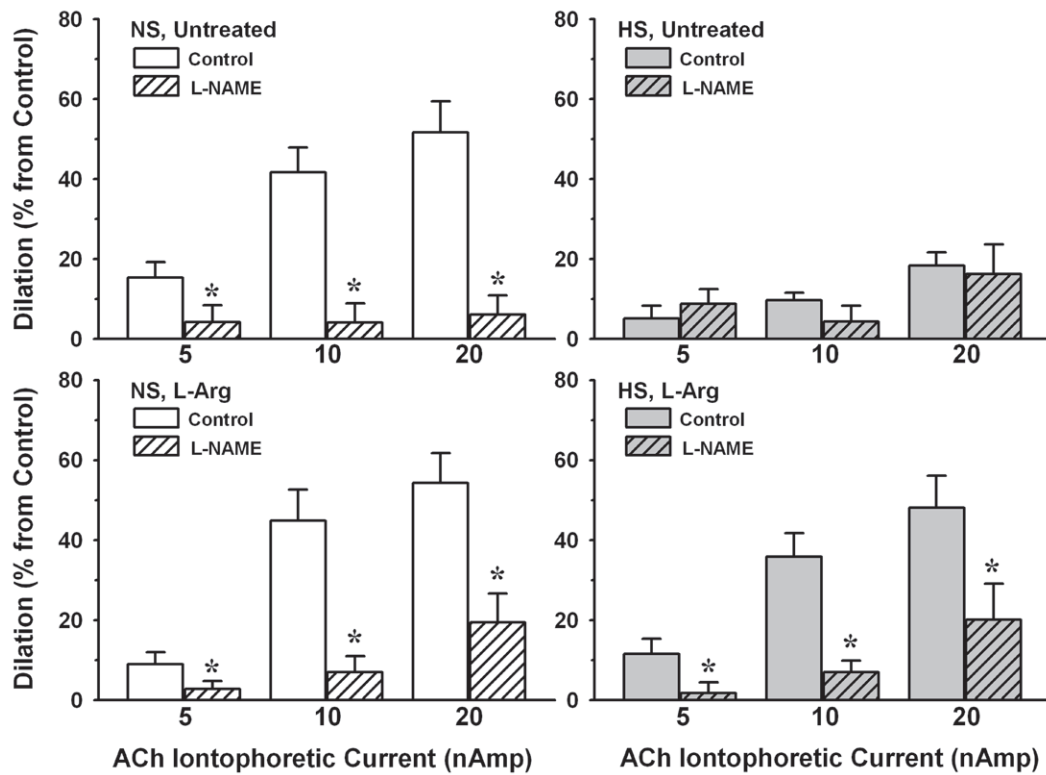


Figure 7. Magnitude of responses to ACh, under normal superfusate (Control) and in the presence of N^ω-nitro-L-arginine methyl ester (L-NAME, 10⁻⁴M), in mice fed NS or HS diet, with or without L-Arg supplementation. Number of vessels for each group same as in Figure 6. * P < 0.05 vs. Control.

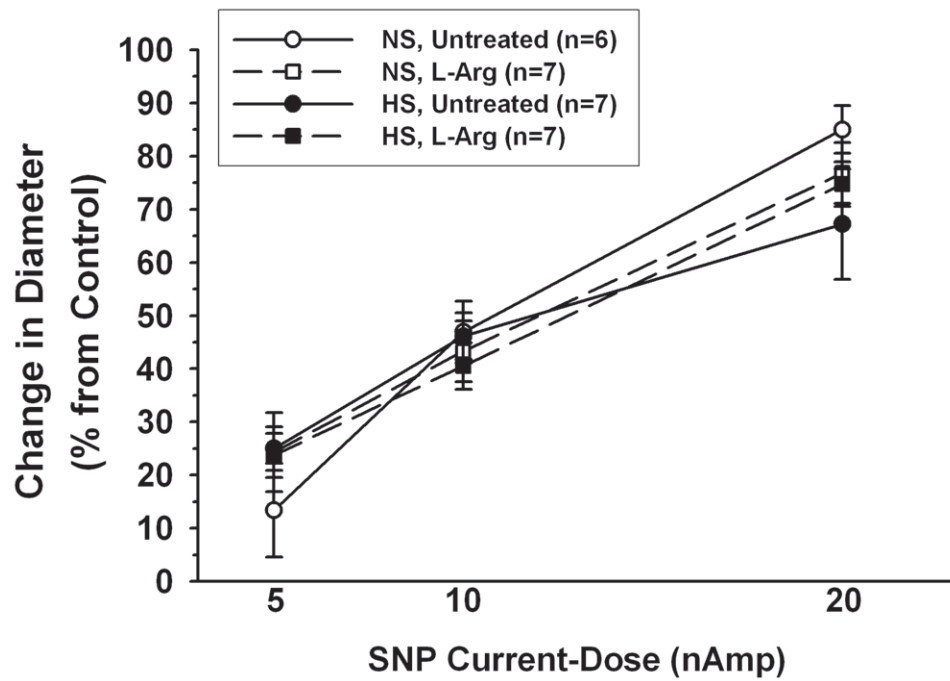


Figure 8. Arteriolar dilation in response to iontophoretic application of sodium nitroprusside (SNP) in mice fed NS or HS diet, with or without L-Arg supplementation. n= number of vessels (1 vessel studied per mouse).